Commentary

Disentangling the MYC web

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That MYC is causally associated with cancer has been apparent for decades. As a retroviral transforming gene or as the target of chromosomal translocations, rearrangements, amplification, mutations, and viral insertions, disturbance of MYC regulation and/or function is one of the most common molecular lesions contributing to multistep carcinogenesis (1–5). Disordered MYC expression alters cell proliferation, cell growth, differentiation, and metabolism. How MYC provokes this panoply of cellular pathology has been debated for years. Whether MYC acts directly on a small number of downstream effectors that elicit many secondary changes or whether MYC itself directly operates on all of these processes has been controversial. The works of Menssen and Hermeking (6) in this issue of PNAS and other recent studies (6–11) point to a complex web of direct MYC targets regulating metabolic flux and information streams through normal and transformed cells. Their work indicates the need for new tools to understand how multiple signals and processes are superimposed and integrated to determine the fate of a cell.

The *c-myc* gene encodes a helix–loop–helix basic leucine zipper protein (HLH-bZIP) that when dimerized with the appropriate partner, binds to the E-box DNA sequence, CACGTG (1–5). E-box-bound MYC interacts with the basal transcription apparatus and with complexes that remodel and modify chromatin. Belying its biological importance, MYC has proven to be only a weak activator or repressor of synthetic reporters and for a few generally accepted natural targets. Because MYC is not a dominating transactivator either *in vivo* or *in vitro*, it has been difficult to sift the true MYC targets from the clutter of low-amplitude changes in gene expression caused by secondary effects.

A variety of strategies and systems have been used to control MYC expression to enable comparison of target gene expression before and after MYC induction (6, 9, 10). Tetracycline-regulated MYC induction as well as retroviral and adenovirus vectors have been used to induce MYC at the RNA level; activation of a MYC-estrogen receptor ligand-binding domain chimeric protein residing in cells eliminated the inevitable delay required to synthesize and translate *c-myc* mRNA, and in principle, revealed MYC targets by means of hormone dependency (12). Whether expressed *de novo* or activated by hormone, exogenous MYC provoked a host of changes; the problem has been to discern the changes spotlighting true MYC-dependent pathways from misleading clues caused by inappropriate and artificial overexpression. (However, during carcinogenesis, overexpression and misexpression of MYC may activate cancer-specific, nonphysiological MYC targets.) The goal has been to relate the physiological and pathological changes after MYC activation or inactivation with the expression of targets.

Whereas early studies of MYC either focused on specific targets implicated in the control of proliferation, growth, differentiation, and apoptosis, recent methods to analyze quantitatively global gene expression changes after maneuvers altering MYC levels and action, coupled with the use of methods to identify endogenous MYC-chromosomal binding site-complexes, promise to identify MYC targets and to define their kinetics of induction and repression. Several studies have used microarray analysis to compare MYC-driven changes in global gene expression (6–11). Although the targets identified in these somewhat overlap, there are also differences, perhaps related to the experimental cell systems studied. (A systematic comparison of reports is hindered by the lack of a standard system and the lack of a convenient, efficient method to compare regulated genes between publications). Menssen and Hermeking use serial analysis of gene expression (SAGE) (13) to quantitate transcript-by-transcript the perturbation in mRNA levels in primary human endothelial cells provoked by adenovirus-directed MYC. At the 95% confidence limit, 216 genes were induced and 258 repressed by MYC, therefore about 5% of all mRNAs seemed to be MYC-responsive. (The application of the 95% confidence limit to thousands of target genes virtually insures numerous false positives and false negatives.) Although cDNA microarray and quantitative real-time PCR analyses on the same RNA samples seconded the candidacy of some targets, the overall concordance of genes induced two-fold or more by SAGE with microarray was about one-third. Clearly additional studies and time are required to define the strengths, limitations, and caveats associated with each of these technologies. Nevertheless, the emergence of such generally accepted MYC targets as ornithine decarboxylase, carbamyl phosphate synthase, and prothymosin (as well as the Adeno-MYC itself) provides reassurance of the overall validity of the approach. To address the issue of whether MYC acts directly at the DNA level, Menssen and Hermeking used chromatin immunoprecipitation (ChIP) to study complexes of cellular MYC with several candidate target genes. With ChIP, formaldehyde cross-links MYC to DNA (or to nearby DNA-bound proteins) while fixing cells. Immunopurification of MYC–DNA complexes from sheared, fixed chromatin with anti-MYC followed by cross-link reversal and PCR with gene-specific primers reveals the presence of genomic sequences bearing the candidate MYC-binding sites. Menssen and Hermeking successfully demonstrated MYC binding at E-boxes in several new MYC targets identified with SAGE. The ChIP-verified targets are otherwise indistinguishable in their degree of MYC responsiveness from the other candidates. Importantly, ChIP verifies the presence of endogenous MYC at the same targets revealed by MYC overexpression. It seems likely that many of the other putative MYC targets will similarly harbor native MYC under some circumstances *in vivo*.

What are the candidate MYC targets and how do changes in their gene expression in response to MYC correlate with dysfunction of major cellular processes? MYC-responsive targets include representatives of virtually every major biochemical and regulatory process in the cell. Few differentiation-specific or "specialty" proteins are seen. The preponderance of targets encodes intracellular proteins. Enzymes and structural proteins associated with the synthesis and degradation of DNA, RNA (transcription, pro-

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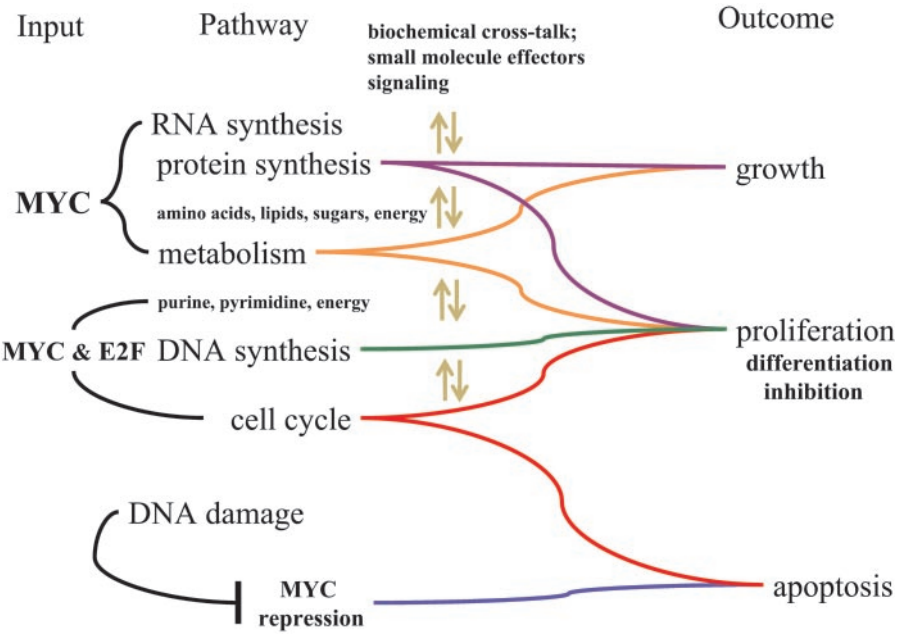

Fig. 1. Pulling different threads of the MYC web activates different combinations of pathways, yielding different outcomes. Other factors such as the E2Fs may shift the relative outcomes. Biochemical cross-talk is undoubtedly required to coordinate pathways. Growth requires activated metabolism more than cell-cycle activation whereas proliferation needs both; proliferation signals delivered in a nonsupportive setting yield apoptosis, etc. DNA damage relieves genes triggering apoptosis from MYC repression (40).

cessing, and transport), protein, carbohydrate, and lipids are all represented. Factors contributing to cell-cycle progression or to apoptosis are also prominent.

A brief synopsis of MYC biology will be helpful to relate MYC targets with MYC function. Cells lacking c-MYC are impaired for growth and proliferation. MYC-haploinsufficient cells double more slowly than their parental cells, and the complete loss of *c-myc* expression in somatic cells leads to severely impeded proliferation (14). Although loss of *c-myc* expression in knockout mice leads to death during early embryogenesis (15, 16), *c-myc*-haploinsufficient mice grow smaller than their wild-type siblings (15). The differential effects of MYC on growth and proliferation have not been resolved. Individual cells lacking MYC may exhibit normal cell-cycle kinetics, but they enter the cell cycle with reluctance and, having little mitotic inertia, readily cease to divide (15, 17, 18). MYC overexpression, misexpression, and deregulation generally increase rates of cellular proliferation and growth, with accompanying inhibition of differentiation, but in some circumstances, elevated MYC provokes apoptosis (4, 5). No single MYC target, validated or proposed, seems to account fully for the biological effects of MYC; none could be related to MYC in a single linear effector pathway or by epistasis (19). A deficiency of Myc can be rescued only by MYC itself or the highly related N-myc oncogene or MYC itself. If no single target suffices to impel MYC action, then clusters of MYC targets must cooperate or conspire to maintain normal physiology or create pathologic mischief (Fig. 1).

In the matter of proliferation, it is tempting to focus on MYC as a cell-cycle regulator through target cyclins and cyclin-dependent kinases (CDKs), CDK inhibitors, etc. (20, 21). Under the lens of MYC biology, however, this focus is blurred. If MYC is a key to regulate mitosis and proliferation, then how does one explain the normal cell-cycle kinetics of some MYC-minus cells? In these cells, MYC may gate the G_0/G_1 transition at cell-cycle entry but thereafter events proceed on schedule (15, 17). MYC-minus cells drop out of the cell cycle with higher frequency than MYC-plus cells, long before the crisis provoked by telomere shortening. The protein component of telomerase, TERT, is an MYC target (22), and it makes sense that disturbed TERT abnormalities may contribute to MYC pathology, but it seems unlikely that the reduced proliferative capacity of cells lacking MYC derives from acute telomere shortening.

The role of MYC as an overseer of proliferation is also dramatized by the inclusion among its targets of proteins and enzymes involved in DNA replication and repair. BRCA1 and MSH2 surface here as MYC targets; however, abnormal DNA synthesis and maintenance alone cannot explain the full role of MYC in cancer. If defective MYC or excessive MYC triggered DNA damage in an oncogenic pathway, then sustained MYC activity would not be required for transformation; reversal of MYC overexpression suppresses growth and even eliminates established MYC-dependent tumors (23, 24). It is difficult to devise a scheme where MYC operates through a single process (Fig. 1).

How MYC regulates growth is only superficially clear. Consistent with the notion that MYC regulates cell size and growth, numerous ribosomal proteins and other molecules associated with protein synthesis are listed as MYC targets. (Menssen and Hermeking also report that several ribosomal proteins are decreased—some of these same proteins, however, have also been reported to be increased by MYC with microarray analysis.) Although some of this response might be attributed to the mitogenic effect of MYC (cells demand more protein to proliferate), MYC is an insufficient stimulus for cell division in the human umbilical vein endothelial cells studied by Menssen and Hermeking. Thus in these cells, MYC expression may prime the protein synthetic apparatus for the production of cellular mass (Fig. 1). Germinal center cells that proliferate extremely rapidly, however, up-regulate neither ribosome synthesis nor MYC (25).

Although there is some disagreement whether MYC regulates cell size or cell number, there is no doubt that the overall mass of a MYC-minus population is reduced. The decision to accumulate biomass within a larger cell or to partition material between daughter cells is unlikely to be made by MYC alone; the linkage between MYC action, protein synthesis, cell growth, and cell division is likely to be highly modulated. If activating protein synthesis is a primary function of MYC, then how does MYC override this aim in the absence of a complete growth signal to accomplish just the opposite—apoptosis with protease activation and the degradation of cellular constituents?

MYC seems to regulate the expression of enzymes involved in a variety of metabolic pathways (Fig. 1). Might the role of MYC be to adjust the flux of metabolites through various catabolic or anabolic pathways? Included among MYC targets are enzymes executing rate-limiting steps and allosterically regulated reactions; it is easy to imagine that these enzymes might control the availability of key nutrients supporting growth and proliferation. It is also possible that the levels of key metabolites serving as allosteric effectors or intracellular signaling ligands might be adjusted to coordinate various cellular processes. Therefore, MYC might serve as a component of an intracellular chemostat helping to set the growth and proliferation potentials of the cell. However, some of the MYC target enzymes catalyze non-rate-limiting reactions with small ΔGs; it is not obvious that minor adjustments in the expression of these genes

would have a significant influence on the physiology or pathology of the cell.

How does MYC exert protean, plastic influence over so many intracellular processes? MYC does not seem to provide a dominating influence on any promoter other than synthetic reporter genes driven by E-boxes. Every promoter characterized as MYC-responsive also recruits other transcription factors. Moreover, other E-box binding proteins may compete with MYC for action at a given site (26, 27). MYC-responsive promoters may integrate input from multiple factors incrementally. In contrast to the situation at the β-IFN promoter/enhanceosome, where the synergistic, synchronous, and choreographed action of multiple factors culminate in a robust all or none response (28), MYC targets are expressed in a more graded manner. Expression of these promoters is likely to be highly sensitive to the biological conditions of the system, according to the panel of sites occupied and the array of factors and cofactors available to cooperate with MYC. Thus, a gene such as CDK4 may respond to MYC in one setting but may be insensitive in transformed cells when driven by other factors. The particular arrangement of sites at target promoters is also an important influence on MYC activity; docking MYC at different distances from the transcription start site modulates its activity (26). Thus, it is seems unlikely that there is a stereotypical response of MYC targets to maneuvers altering MYC levels. At different promoters MYC may act through different mechanisms and at different stages of the transcription cycle (29). At some promoters where MYC operates conventionally opening chromatin and recruiting the basal transcription machinery, binding to E-boxes precedes the recruitment of RNA polymerase. Elsewhere, MYC is recruited to promoters bearing prebound RNA polymerase II. In this situation, MYC is likely to control stages of the transcription cycle after preinitiation complex formation, such as promoter clearance and escape. MYC action at targets has been associated with histone acetylation, most dramatically with H4 modification (30–32). Chromatin modification occurs throughout the transcription cycle, and the ability of MYC-recruited complexes to orchestrate or influence the sequential steps of activation or repression will almost certainly be context- and situation-dependent (33–35). The MYC response will be plastic determined by the input from other factors and signals.

Superimposed on the complexity of MYC action and targets is the problem of *c-myc* expression. Controlling where, when, and how much MYC is made determines much of its action spectrum. For example, the structural differences between N-MYC and c-MYC are less important than the differences in their transcriptional regulation. Rescue of early embryonic lethality in *c-myc* knockout mice by substituting N-myc into the disrupted locus dramatizes the importance of a proper *c-myc* promoter function (36). The *c-myc* promoter responds to numerous signals and transcription factors. It seems likely that *c-myc* will respond to feedback from the different systems regulated by MYC. The mechanisms integrating this diverse and dynamic input are not understood.

The answer to the question—"What are the targets of MYC?"—is at hand. The diversity and plasticity of the MYC response highlights the next challenge: to understand how MYC administrates the molecular protocols linking its subordinate molecular subsystems into a physiological functioning unit. To accomplish this, approaches for manipulating genes in sets and combinations will be necessary. Defining groups of MYC targets recapitulating particular features of MYC biology as well as teaming MYC with other broadly acting transcriptional regulators (such as the E2Fs) may define regulatory and effector subassemblies. RNAi (37) and improved strategies for knocking out genes rapidly (38) will help to study the interactions of MYC with other gene regulators and among MYC targets, much as the use of synthetic lethal mutations in yeast have provided a window to structural, regulatory, and catalytic networks. The use of high-throughput mass spectrometry to identify the components of macromolecular clusters will also help to reveal the interactions among the regulators, partners, and targets of MYC (39). We have developed the physical tools to explore the proteome and genome. We await a theoretical framework to link the components and make quantitative predictions for the MYC web and other networks.

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